International Journal of Pharmacy and Natural Medicines

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RESEARCH ARTICLE

Antioxidant, Hepatoprotective Activity and RP-HPLC Analysis of *Moringa Oleifera* Pods Methanolic Extract against Alcohol Induced Hepatotoxicity

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ABSTRACT

In this study the pods of *Moringa oleifera* were extracted with methanol and subjected to analysis of the polyphonolic compounds by RP-HPLC method and to appraise the free radical scavenging activity and hepatoprotective activity aligned with the injury caused by alcohol to liver in Wister albino rats. Polyphonolic compounds like quercetin, gallic acid and rutin were seen in RP- HPLC study. The extracted *Moringa Oleifera* pods in methanol and ascorbic acid (standard) was subjected *to in vitro* free radical protective activity by inhibition of lipid peroxide, superoxide, hydroxyl and DPPH (2,2-diphenyl-1-picryl hydrazyl) radical. The *in vivo* antioxidant and hepatoprotective effect of *Moringa oleifera* pods which was methanolic extract was investigated at selected doses of 100, 200 and 400 mg/kg body weight per day, and standard drug Silymarin at dose of 100 mg/kg body weight per day were administered for 25 days by oral route. The ethanol (40%) used as a hepatotoxicant and dose selected for liver injury was 3.76 gr/kg body weight per day given orally for 25 days. The IC_{50} values of methanolic extract of *Moringa Oleifera* pods was found to be 256.50µg/ml for super oxide radical scavenging activity, 287.50µg/ml for DPPH radical scavenging activity, 363.30µg/ml for hydroxyl radical scavenging activity and 805.41µg/ml for inhibition of lipid peroxidation. Ethanol treatment in rats caused severe liver damage indicated by marked elevated liver biomarkers like serum aspartate aminotransferase (SGPT), alanine aminotransferase (SGOT), alkaline phosphatase (ALP), total bilirubin and physical parameters like liver weight and liver volume, as well as pronounced decreased in serum total protein compared to normal rats. Oxidative stress status was appeared on alcohol control evident as recorded significant dwindling in levels of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), concurrence with remarkable amplification on oxidative stress marker; malondyaldehyde (MDA) concentrations with respect to normal values. The *Moringa oleifera* pods which was extracted in methanol and standard Silymarin on oral administration significantly protect the liver from toxic dose of ethanol on the above serum liver biomarkers, antioxidant and physical parameters in curative model. All these palpable results were confirmed by histopathological observations, which indicated that the presence of polyphonolic compounds in methanolic extract of Moringa *oleifera* pods showed normalization of degenerated and fibrotic liver tissue as of alcohol treated group.

Keywords: *Moringa oleifera* pods, RP-HPLC, Antioxidant, Alcohol, Hepatoprotective.

ARTICLE INFO

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MS-ID: IJPNM3450

PAPER QR-CODE

ARTICLE HISTORY: Received 28 August 2018, **Accepted** 19 October 2018, **Available Online** 15 December 2018

Citation: A. Prathyusha. Antioxidant, Hepatoprotective Activity and RP-HPLC Analysis of *Moringa Oleifera* Pods Methanolic Extract against Alcohol Induced Hepatotoxicity. *Int. J. Pharm. Natural Med.,* 2018, 6(2): 147-158.

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1. Introduction

Consumption of alcohol in India is one of the major causes of preventable mortality. It is also the same worldwide. Liver diseases are caused by drinking alcohol which leads to health problems. Excessive intake of alcohol can damage almost all organs of the human body (Charness ME et al, 1986). Ethanol oxidation takes place in the liver and that is why this organ is prone to damage (Geokas MC et al, 1986). Susceptible changes in the liver are seen because of chronic alcohol intake which leads to cirrhosis, fibrosis, steatosis and alcoholic hepatitis. The toxic effects of acetaldehyde are generated by alcoholic metabolism which leads to alcoholic injury (Hoemera M et al, 1988). The protein coding gene CYP2E1 by ethanol generates free radicals. Hepatic damage is caused by free radicals. The protein coding gene CYP2E1 induces hepatocytes by ethanol, increasing free radical production, which in turn accounts for alcohol induced liver injury (Knechakt et al, 1995). There is enough evidence to support the earlier hypothesis that kuffer cells play an important role in alcohol induced liver disease because of the resident hepatic macrophages (Nolan jp et al, 1980).

Moringa oleifera is a tree which has lots of medicinal values such as antispasmodic, stimulant, expectorant and diuretic. It also has healing properties. In the Vedic literature in India, 5000 years ago its medicinal properties were documented (Patwardhan B, 2000). The tree of *Moringa oleifera* grows very fast and reaches a height 10 or 12 m. Its fragile branches keep spreading providing feathery foliage of tripinnate leaves and a whitish gray bark. The root has a taste similar to that of horse-radish and is acrid. It has stimulant, diuretic and antilithic action when consumed internally. The gum derived from the plant is bland and mucilaginous. The plant seeds have pungent and refreshment properties. The plant bark has abortifacient, antifungal, antibacterial and emmenogogue effects. The flowers of the plants increase the flow of the bile apart from cholagogue, diuretic and stimulant action. It is also antiseptic and cardiac circulatory tonic (Nadkarni KM, 2008). Fried pods of Moringa *oleifera* are useful in hepatic and diabetes disorders. Antihelmintic and antipyretic activity was seen in regular pods. They are also used as a diuretic in calculus affection apart from nervous encumbrance, distended liver and spleen, asthma and deep seated inflammation. Decoction of the pods is useful in hoarseness and sore throat when used as a gargle. Antiparalytic properties were seen in root and fruit. Juice derived by crushing the leaves of the *Moringa oleifera* plant can treat hiccough (in high doses it is emetic). When these leaves are cooked they treat influenza and catarrhal afflictions. Bark obtained from the root has antiviral, anti-inflammatory and analgesic properties. Bark obtained from the stem and flowers are hypoglycemic. When the seeds are infused they produce anti-inflammatory, antispasmodic, diuretic and can also be used in venereal diseases. The dried seeds were indicated in glycosuria, goiter and lipid disorders. The leaf, seed, root and stem bark of the plant indicated in internal abscess and piles (Khare CP, 2007). These are some of the therapeutic applications mentioned in the Ayurvedic Pharmacopoeia of India. Potent native herbal medicines for liver disorders were available in various parts of the world but have not been validated scientifically. If this validation is done it will lead to the blooming of cost effective drugs. This study revolves around methanolic extract of *Moringa oleifera* pods for hepatoprotective activity against alcohol induced hepatic damage in animal studies.

2. Materials and Methods

Chemicals and drugs

The various chemicals and drugs which were used in this study were purchased and obtained as gift samples.

- Purchased estimated kits of Erba diagnostics from local suppliers, in Guntur, India. Such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBR) and total proteins (TP).
- Purchased Nitrobluetetrazolium (NBT) from Sisco research labs Pvt Ltd, Mumbai.
- Purchased 2-deoxy-D-ribose and 2, 2-diphenyl-1-picrylhyrazil (DPPH) from sigma chemicals.
- We received a gift sample of Silymarin from micro labs, Bangalore, India.

The remaining chemicals and reagents which were used in this study were of analytical grade.

Analysis of polyphonolic compounds of *Moringa oleifera* pods extract by RP-HPLC

At room temperature one gram fine powder of *Moringa oleifera* pods which was dry, was extracted overnight in 20 ml methanol. The extracted supernatant which was then filtered and latter vacuumed dried for optimistic biomass recovery and latter dissolved in 2 ml of methanol. The filtration done was done using 0.22 µm filter (Milipore, USA). The recovered biomass was screened for polyphonolic compounds by using RP-HPLC method. The detection of polyphonolic compounds like quercetin, gallic acid and rutin were done under following conditions (Pravej Alam et al., 2016):

Instrument: Shimadzu
Detector: SPD-20A Prominence UV-VISIBLE detector
Mobile Phase: Methanol: Acetonitrile (60:40)
Column: Enable Make C18G (250 X 4.6 mm; 5µ)
Flow rate: 1ml/min
Injected volume: 20µl
Wave length: 225nm

Preparation of extract

The *Moringa oleifera* pods under investigation were coarsely powdered using a mechanical grinder, sieved by 60-80 mesh size. The dried powdered *Moringa oleifera* pods selected for the study were weighed (1kg) and packed in a Soxhlet apparatus. At room temperature the fine powder of plant material was extracted with 95% of methanol (1 liter) in Soxhlet apparatus for 72 hrs. The macerated plant powder was filled in a soxhlet apparatus for continuous extraction with 3 liter of 95% of methanol. To remove the all traces of the solvent present in the extract was dried in rotary evaporator (Rajkiran K *et al.,* 2015).

In vitro* antioxidant activity*: The *in vitro* antioxidant activity of methanolic extract of *Moringa oleifera* pods was evaluated by Superoxide radical protective activity (Robak and Gryglewski *et al*., 2007), lipid peroxidation inhibition activity (Ohkawa *et al*., 1979), hydroxyl radical protective activity (Elizabeth and Rao., 1990) and DPPH radical protective activity (Braca *et al*., 2003).

Acute oral toxicity study (LD_{50})

Before the experiment, all the albino mice which weighed between 25 and 40 g were on an overnight fasted. The up and down procedure LD_{50} was done adopting the method of Bruce (Bruce RD., 1985). The oral administration of selected doses of extract was 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 g/kg body weight. Food was withheld for next 3-4 hours after administration of plant methanolic extract. Periodic observation of the animals were for toxic symptoms were done continuously, initially for the first 30 minutes after dosing and thereafter special attention given during the initial 4 hours, there on for next 24 hours and daily for 14 days. For compute the toxicity changes in the skin and fur, eyes and mucous membrane were observed and we also noticed behavioral parameters in mice. After oral administration of various doses of extract and that indicting their lethal dose (LD_{50}) of the extract which was 5.0 g//kg body weight showed absence of toxic symptoms.

Experimental animals

Mahaveer enterprises, Hyderabad, India, supplied Albino rats Wistar strain weighing 180 ± 20 g. These rats were kept in our animal house at the desired temperature of (23 ± 2)^{o}C with a humidity of 50-60 % was maintained. Also, the darkness and light cycle of 12 hours: 12 hours was considered. They were fed sumptuously with pellet diet and water at regular intervals. The research lab has approval from CPCSEA, Govt. of India (Regd. No. HCOP/IAEC/PR-2/2018) and the conducted study was in accordance with the Institutional Animal Ethics Committee.

Experimental design for *in vivo* hepatoprotective activity (K Eswar Kumar *et al.,* 2013) After 48 h of acclimatization, prior to experimental study all animals were adapted to laboratory conditions. In our study, 36 rats were used which were randomly divided in to 6 groups and every group have 6 rats. The plan of work as follows:

Group A: Received only 1% w/v sodium CMC orally for 50 days.

Group B: Received Ethanol 3.7 g/kg as toxicant orally for 25 days followed by 1% sodium CMC from 26 to 50th day.

Group C: Ethanol 3.7 g/kg orally for 25 days followed by methanolic extract of *Moringa oleifera* pods 100 mg/kg orally from 26th Day to 50th day.

Group D: Ethanol 3.7 g/kg orally for 25 days followed by methanolic extract of *Moringa oleifera* pods 200 mg/kg orally from 26th Day to 50th day.

Group E: Ethanol 3.7 g/kg orally for 25 days followed by methanolic extract of *Moringa oleifera* pods 400 mg/kg orally from 26th Day to 50th day.

Group F: Ethanol 3.7 g/kg orally for 25 days followed Silymarin 100 mg/kg orally from 26th Day to 50th day.

Biochemical estimation

In this study Silymarin (standard), alcohol (toxicant) and different doses of plant extract were given orally. Blood samples from retro-orbital plexus were collected on the 0th, 26th and 51th day. The blood samples were then centrifuged at 3000 rpm for 20 min to obtain clear serum, which were used for the assessment of hepatic biomarkers such as ALP, AST, ALT, total bilirubin and total protein by using semi autoanalyzer. The rats were scarified after collection of blood samples and the liver was isolated from each rat for assessment of physical parameters, antioxidant enzymes activity and histopathological study.

***In vivo* antioxidant enzymes activity**

The isolated livers were rinsed in ice-cold normal saline, followed by 0.15M Tris-Hcl buffer pH 7.4 and later weighed after being bloated dry. For estimation of lipid peroxidation a 10% w/v of homogenate was prepared in 0.15 M Tris Hcl buffer (Ohkawa H et al., 1979). After precipitating the proteins with trichloroacetic acid a part of homogenate was used for estimation of glutathione peroxidase (Ellman GL., 1959). The balanced homogenate was centrifuged at 15000 rpm for 15 min at 4^{o}C. The estimation of superoxide dismutase and catalase were from the supernatant obtained by centrifugation process (Mishra HP and Fridovich I., 1972 and Aebi H., 1984).

Calculation of Percentage Hepatoprotection (Rajkiran K *et al.,* 2017)

The percentage of hepatoprotection offered by the different doses of methanolic extract of *Moringa oleifera* pods and Silymarin was calculated by following formula.

$$H = \left(\frac{T-B}{T-C}\right) \times 100$$

Where T = Mean value of hepatic biomarkers produced by ethanol treatment

B = Mean value of hepatic biomarkers produced by plant extracts and ethanol treatment C = Mean value of hepatic biomarkers produced by control group

H = Percentage of liver protection

Statistical analysis

The experimental results were indicated as Mean± S.E.M (n=6). The data was subjected to analysis of variance (one way ANOVA) to determine the significance of changes

followed by Tukey multiple comparison test using Graph Pad Prism-5 software. P value < 0.05 was considered to be statistically significant. # indicates P <0.05, ## indicates P<0.01 and ### indicates P<0.001 as compared Group B with normal control group (Group A); *indicates P<0.05, **indicates P<0.01 and ***indicates P<0.001 as compared rest of groups with Group B treated with alcohol and ns = not significant when rest of Groups compared with Group B.

3. Results and Discussion

Analysis of polyphonolic compounds of *Moringa oleifera* pods extract by RP-HPLC

RP-HPLC analysis of the *Moringa oleifera* pods methanolic extract indicated the presence of:

1. Quarecetin, with retention time (4.151) minutes, Figure (1) in comparison with standard (4.171) as Figure (2).
2. Gallic acid, with retention time (3.406) minutes, Figure (1) in comparison with standard (3.222) as Figure (3).
3. Rutin, with retention time (3.092) minutes, Figure (1) in comparison with standard (3.147) as Figure (4).

***In vitro* antioxidant activity**

Superoxide scavenging activity

Super oxide is biological important since it can be decomposed to form strong oxidation species such as singlet oxygen and hydroxide radicals is very harmful to the cellular components in a biological system. In superoxide scavenging activity, assay the super oxide anions were generated by using PMS – NADH – system. Ascorbic acid was used as control and different concentration of *Moringa Oleifera* pods methanolic extract was evaluated for antioxidant potential. The 50% inhibition (IC_{50}) values of Ascorbic acid recorded was 108.83 µg/ml whereas the 50% inhibition values recorded for methanolic extract of *Moringa oleifera* pods was 256.40 µg/ml (Table 1& Histogram 1).

Inhibition of lipid peroxidation activity

Induction of Fe^{2+}/Ascorbate generates the lipid peroxides. The different concentrations of plant extract and ascorbic acid was found to be dose dependent inhibition of lipid peroxides (Table No.1 & Histogram No. 1). The concentration of methanolic extract of *Moringa Oleifera* pods and ascorbic acid needed for 50% inhibition (IC_{50}) of lipid peroxide radical was found to be 805.41µg/ml and 461.50µg/ml respectively.

Hydroxyl radical scavenging activity

Hydroxide radical scavenging activity is also most biological importance because the degradation of deoxyribose mediated by hydroxyl radical generated by Fe^{3+}/Ascorbate/EDTA/H_2O_2 system was found to be inhibited by the extracts. The different concentrations of methanolic extract of *Moringa Oleifera* pods and ascorbic acid was found to be dose dependent inhibition of lipid peroxides. The concentration of methanolic extract of *Moringa oleifera* pods and ascorbic acid needed for 50% inhibition (IC_{50}) of hydroxide radical was founded. The IC_{50} values of ascorbic acid was 284.91µg/ml whereas the 50% inhibition values recorded for methanolic extract of *Moringa oleifera* pods was 363.30 µg/ml (Table No.1 & Histogram No. 1).

DPPH radical scavenging activity

To explore the protective activities of several natural compounds widely used *in vitro* antioxidant scavenging activity is DPPH radicals. DPPH (Para magnetic compound) with an odd electron shows strong absorption band at 517nm in methanol. The absorbance decreases due to colour change from purple to yellow because of the scavenging the free radical by antioxidant through donation of hydrogen to form the stable DPPH-H molecule. In DPPH assay, ascorbic acid was used as control and different concentrations of methanolic extract of *Moringa Oleifera* pods were evaluated for antioxidant potential. IC_{50} values of ascorbic acid recorded were 157.45µg/ml. Whereas IC_{50} values recorded for methanolic extract of *Moringa Oleifera* pods was 287.50µg/ml (Table No.1 & Histogram No. 1).

Alcohol induced intoxication (Curative study):

Estimation of Serum liver biomarkers and physical Parameters

Based on the levels estimated before the treatment in various group rats, the normal variation in liver biomarkers (AST, ALT, ALP, Total Bilirubin and Total Protein) and physical parameters such as liver weight and liver volume were calculated. The results are given in Table no 6-7. The normal variation in SGPT, SGOT, ALP, Total Bilirubin and Total Protein levels in the experimental animals (n=36) was found to be in the range of 52.04-67.23 U/L, 82.23-90.93 U/L, 140.28-160.18 U/L, 0.27-0.34 U/L and 7.86-8.94 U/L respectively. The normal variation in liver weight and liver volume in the experimental rats (n=6) was found to be in range of 3.24-3.58 g/100g and 3.30-4.01 ml/100g respectively. The rats in Group A (control) treated with 1% sod. CMC showed no change in the serum liver biomarkers and physical parameters and lies within the normal range.

The Group B rats treated with 40% alcohol for 25 days showed significant (p<0.01) increased level of ALT (219.25±2.01), AST (301.17±5.36), ALP (389.24±6.42), total bilirubin (5.79±0.38), liver weight (7.08±0.12) and liver volume (7.48±0.15), where as serum total protein (3.31±0.38) levels significantly (p<0.01) decreased when compared with normal treated group rats. The results were given in table no. 2-4.

Group C, D, E and F treated rats showed significant (p<0.01) decrease in serum liver biomarkers (AST, ALT, ALP and Total Bilirubin) and physical parameters (liver weight and liver volume), where as serum total protein levels significantly (p<0.01) increased when compared to alcohol treated rats (Table.No.2 to 4). Based on SGPT, SGOT, ALP, T.BIL, TPR, liver weight and volume levels the animals treated with methanolic extract of *Moringa Oleifera* pods at a dose of 100 mg/kg, produced 23.37, 16.69, 26.00, 31.26, 28.65, 37.29 and 40.68 % protection respectively. Whereas animals treated with methanolic extract of *Moringa Oleifera* pods at a dose of 200 mg/kg produced 59.77, 45.91, 61.79, 55.03, 44.84, 74.59 and 62.15 % protection and 400 mg/kg produced 78.45, 87.55, 85.60, 87.02, 69.40, 83.98 and 90.11 % protection against alcohol intoxication (Table No.5 & Histogram No. 2).

Based on SGPT, SGOT, ALP, T.BIL, TPR, liver weight and volume levels, the percentage protection was offered by Silymarin found to be 89.84, 94.07, 93.96, 96.89, 79.89, 93.09 and 94.07 % respectively against alcohol intoxication (Table No.5 & Histogram No. 2).

***In vivo* antioxidant activity**: Free radicals are generally produced as a consequence of normal metabolism and due to certain diseases conditions. Superoxide dismutase, catalase and glutathione peroxidase are some important body defence mechanisms to controls the different free radicals in our body due to biological reactions.

Glutathione peroxidase, Super oxide dismutase and Catalase: Chronic exposure to ethanol decreased the activities of certain enzymes (ROS scavenging) such as Super oxide dismutase (SOD), Glutathione peroxidase (GPx) and Catalase (CAT), was revealed in our study. There is an assumption by Sandhir R and Gill KD., 1999 suggesting that there is a decrease in antioxidant activity of enzymes like GPx, SOD and CAT after exposure to ethanol which may be due to the damaging effects of free radicals, or they could be due to the effect of acetaldehyde which is formed by oxidation of ethanol on these enzymes. In our study, it was found that methanolic extract of Moriga *Oleifera* pods could renovate the activity of these antioxidant enzymes and could possibly lower the production of free radicals and hepatocellular damage. (Table No.6 & Histogram No. 3)

Lipid Peroxidation

The formation of Reactive oxygen species (ROS) leading to oxidative stress and hepatocellular injury could be the cause of alcoholic liver disease. Documentation that Kupffer cells are instrumental in formation of ROS during chronic ethanol consumption, they are primed and activated for enhanced formation of pro-inflammatory factors. Increase in lipid peroxidation is due to alcohol induced liver injury. Methanolic extract supplementation of *Moriga Oleifera* pods in this study was effective in blunting lipid peroxidation which was suggestive that the methanolic extract of *Moriga Oleifera* pods due to the presence of polyphonolic compounds (Quercetin, Gallic acid and Rutin) had antioxidant property to decrease ethanol-induced membrane lipid peroxidation (Table No.6 & Histogram No. 3).

Histopathological studies

In curative study by the methanolic extract supplementation Moringa oleifera pods which offered hepatoprotection to provide biochemical, antioxidant and physical evidence which were seen in the results of the histopathology study. Normal hepatic cells are polygonal and were binucleated with nucleolus and abundant cytoplasm (Naidu RS et al., 2007). In Normal control group (Group A) hepatic globular structure was found to be normal and no abnormality was seen. But in alcohol treated group (Group B) the histopathology revealed derangement of cords, fatty ballooning, microvasicular steatosis, fatty and vaculolar degeneration, cell enlargement, cellular infiltration were seen without any regeneration (Fig.No.1), however, methanolic extract of *Moringa Oleifera* pods reduced these abnormalities in dose dependant manner with hepatic regeneration (Fig. No.1).

Discussion

Heavy and long term use of alcohol and consumption of alcoholic beverages increases the risk of alcoholic liver disease in nearly all the organ systems of the human body. The three major enzyme systems that mediate the commencement of ethanol metabolism (oxidation) in the liver, however, the main system is alcohol dehydrogenase (ADH), the cytochrome P450 systems (primarily CYP2E1) and catalase play important roles. Acetaldehyde is produced by all three biochemical pathways as the end product. Acetaldehyde which is later oxidised inti acetate aldehyde dehydrogenase (ALDH), this reaction is slow and the kinetics do not allow the accumulation of detectable increases in acetaldehyde in humans who consume alcohol (Arteel G et al., 2003).

The toxicity of the major metabolite acetaldehyde is seen in alcohol induced tissue damage. In chronic liver diseases the increased formation of ROS such as hydrogen peroxide and superoxide anions are the main factors (Khan AJ et al., 2009). An increase in pro-oxidant formation is not the lone reason for oxidative stress associated with clinical Alcoholic Liver Disease. A decrease in overall alcoholic antioxidant status along with lower levels of key dietary antioxidant molecules is seen in Alcoholics. In ALD oxidative stress is mostly caused by both an increase in pro-oxidant production and a decrease in anti-oxidant defences (Arteel G et.al., 2003). Alcohol induced liver injury can be accounted hypothetically is that CYP2E1, induced increased production of free radicals in hepatocytes by ethanol. A rich source of micronutrients, vitamins, antioxidant, protein and flavonoids are found in Moringa oleifera. In medicine flavonoids are group of polyphonolic compounds were widely used and around 300 known varieties of flavonoids were found in plant kingdom. Anti-inflammatory, antihepatotoxic, antiulcer, antiallergic, antidiabetic and antiviral activities were seen in Rutin and quercetin. Antifungal, antiviral and antioxidant properties are seen in Gallic acid. Gallic acid is useful against oxidative damage of our cells (Pravej Alam et al., 2016). Presences of Rutin, quercetin and gallic acid were seen in the results of RP-HPLC analysis.

The liver damage by alcohol was observed by increase in the serum levels of SGPT, SGOT, ALP, total bilirubin and a decrease in serum total protein and increase in the physical parameters like liver weight and liver volume. Necrosis or membrane damage releases these enzymes into circulation and hence can be measured in serum and these pathological changes also increase the liver weight and volume.

Results of the study clearly demonstrated that the serum level of hepatic enzymes AST, ALP, ALT, total bilirubin were increased, and Total Protein levels were decreased in the present study, reflecting the hepatocellular damage in the alcohol induced hepatotoxicity in animal model. The different doses (100, 200 and 400mg/kg) of *Moringa Oleifera* pods methanolic extract considerably ($p<0.05$ to $p<0.01$) decrease the SGPT, SGOT, ALP, total bilirubin, liver weight and liver volume, whereas total protein levels

were increased in alcohol intoxicated rats. Among these doses 400 mg/kg body weight offered maximum recovery. From the results it was found that rats treated with ethanol showed a marked decrease in activities of Catalase and Super oxide dismutase when compared to normal control group. The rats pretreated with Silymarin and Methanolic extract of *Moringa oleifera* pods the activities of Catalase, glutathione peroxidase and Super oxide dismutase had significantly increased when compared to toxicant group. *In vivo* lipid peroxidation study revealed that ethanol treated group showed significant increase in Malondialdehyde (MDA) level when compared with normal control group. Methanolic extract of *Moringa oleifera* pods and Silymarin were able to significantly prevent this rise in MDA level.

The results of biochemical parameters, physical parameters and antioxidant parameters were correlated to the histopathological changes from photomicrographs of rat liver. In alcohol treated group (Group B) showed centrilobular hepatic necrosis, degeneration of hepatic cords and infiltration of lymphocytes. Different doses of methanolic extract of *Moringa Oleifera* pods treatment decrease the histopathological changes due to administration of alcohol. Based on the results the shielding effect of the *Moringa Oleifera* pods methanolic extract against alcohol induced hepatotoxicity may due to the inhibition of hepatic damage and hepatic function markers. The presence of flavonoids like Rutin, quercetin and gallic acid in *Moringa Oleifera* pods methanolic extract responsible for the antioxidant activity (Hussain M et al., 2007; Kumar S et al., 2008) and same might be the reason for the useful activity against hepatotoxicity due to alcohol.

The observations from the present study indicated that when the animals were exposed to two durations of agarwood smoke, the general behavior remained almost same as like the control except the parameters such as alertness, aggressive postures, fights, spontaneous movements that were found to be reduced(Table-1). In these groups the animals remained calm, less agitated and confined to one corner that suggests the smoke exposure might have slowed the alert-induced activities. This is one of the characteristics of aromatherapy where exposure to certain chemicals calms the brain functions leading to relaxations of body activities[2]. Similar findings of inhalation of agarwood oil vapor have shown sedative effect is reported by Takemoto et al., 2008 [15].

The CNS activity were studied by light/dark chamber and elevated plus maze apparatus. In the light/dark test, the data from the table indicated that compared to control group, the 30 minutes exposure of agarwood smoke significantly decreased ($p<0.001$) the number of crossing and decreased ($p<0.05$) the time spent in light chamber and on the other hand the 60-minutes exposure significantly increased ($p<0.001$) the number of crossing, decreased ($p<0.05$) the time spent in dark chamber and increased ($p<0.05$) the time spent in light chamber compared to control (Table-2). Similarly, in the elevated plus maze experiment, the observations indicated that 30-minutes exposure of agarwood smoke did not produce any significant change in the time spent in open and close arms but reduced ($p<0.05$) the number of head dips compared to control. The 60 minutes agarwood smoke exposure enhanced ($p<0.001$) the time spent in open arm, close arm ($p<0.05$) and increased ($p<0.001$) number of head dips without affecting the number of vertical rearing compared to control values (Table-3).

The data suggests that longer duration of agarwood exposure might enhance the explorative action due to suppression of anxiety and its related parameters and shorter duration has opposite action. Compounds exhibiting both anxiogenic and anxiolytic effects have been reported for centrally acting agents such as diazepam and buspiron. Several factors like dose, duration of exposure, mental condition of the subjects, etc are known to play an active role in such types of actions [16]. Since volatile oils present in the agarwood are found to effect the levels of brain neurotransmitters such as serotonin, endorphin and nor-epinephrine[1,3], similar mechanisms could be responsible for dual action of agarwood on the experimental animals when exposed to two durations of smoke. In the reproductive behavior studies, female rats in the estrus phase were allowed with a male rats and their behavior 30 minutes after sunset and 30 minutes before sunrise is recorded[14]. Our observation indicated that the control animals had an average five number of mounts and one intromission and in comparison, the 30 minutes duration exposure increased the number of mounts to eight ($p<0.001$) compared to control and the intromission remained at one. The 60 minutes exposure rats exhibited decreased number of mounts to three ($p<0.001$) and no intromission took place (Graph-1). Studies in the past have reported that over excitation of brain center can adversely affect the reproductive behavior in both male and female sexual arousal [17,18].The brain neurotransmitters particularly serotonin is reported to play a complex role in the excitation of CNS and sexual-related neuropsychology [19]. Since, agarwood is known to elevate the levels of brain neurotransmitters [1,3], similar mechanisms might have occurred for variation in both the CNS and reproductive behaviors due to differences in the duration of smoke exposure.

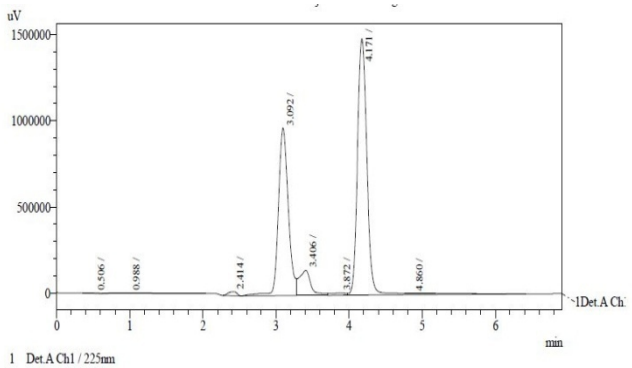


Figure 1: RP-HPLC analysis of Moringa Oleifera pods methanolic extract

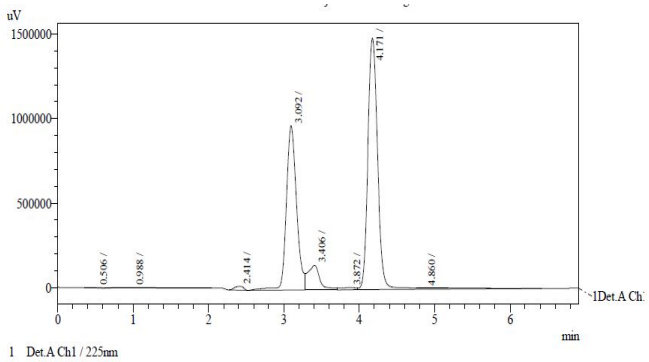


Figure. 2: RP-HPLC analysis of qurecetine standard

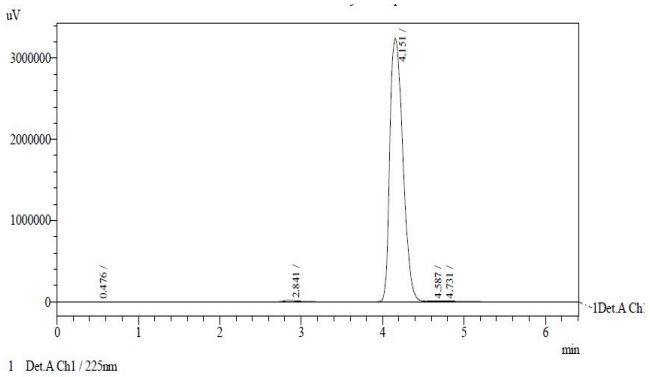


Figure.No.3: RP-HPLC analysis of rutin standard

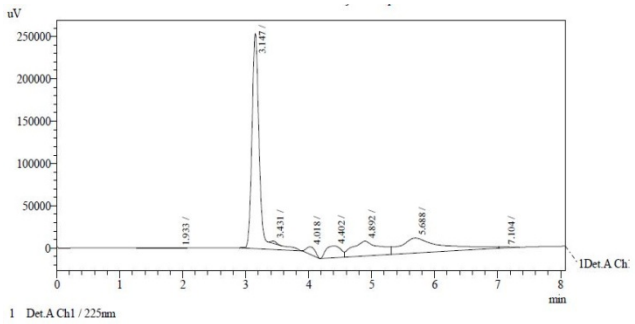


Figure.No.4: RP-HPLC analysis of gallic acid standard

4. Conclusion

For the first time, the hepatoprotective action of the *Moringa Oleifera* pods methanolic extract was as per we observed in this research. The clinical impact of this study is the possession of hepatoprotective activity effect of by *Moringa Oleifera* pods methanolic extract which is attributed to its ability to reduce the rate of lipid peroxidation, enhance the antioxidant defence status and guard against the pathological changes of the liver induced by alcohol intoxication. The strong *in vivo* antioxidant activity also suggests that a dietary supplement of *Moringa Oleifera* pods vegetable can be exploited as a naturally available alternative for drugs supplement development in the prevention and treatment of liver damage

Table 1A: Percentage inhibition of *in vitro* free radical scavenging activity of *Moringa Oleifera* pods methanolic extract and ascorbic acid.

Conc. (µg/ml)	% Inhibition of free radical scavenging activity			
	Super oxide radical scavenging assay		Hydroxyl radical scavenging assay	
	Ascorbic acid	*Moringa oleifera* pods	Ascorbic acid	*Moringa oleifera* pods
25	28.15±0.46	15.29±0.55	17.18±0.68	14.21±0.25
50	40.19±1.07	36.32±0.65	35.92±0.54	32.35±0.57
100	56.78±0.26	52.85±0.29	50.34±0.28	47.98±0.43
250	72.98±0.69	65.51±0.26	60.00±1.01	52.04±0.58
500	80.75±0.46	71.28±0.32	68.54±0.74	65.72±0.64
1000	86.42±1.32	78.94±0.65	80.05±0.69	74.94±0.73
IC_{50}	**108.83**	**256.40**	**284.91**	**363.30**

Table 1B: Percentage inhibition of *in vitro* free radical scavenging activity of *Moringa Oleifera* pods methanolic extract and ascorbic acid.

Conc. (µg/ml)	% Inhibition of free radical scavenging activity			
	Lipid peroxidation assay		DPPH radical scavenging assay	
	Ascorbic acid	*Moringa oleifera* pods	Ascorbic acid	*Moringa oleifera* pods
25	14.29±0.26	5.81±0.27	25.95±0.32	15.95±0.35
50	30.24±0.84	10.57±0.53	45.95±0.38	26.30±0.58
100	44.87±0.36	19.54±0.63	62.75±0.24	56.29±0.51
250	53.57±0.51	30.28±0.55	73.29±0.21	65.28±0.89
500	59.67±0.95	38.94±0.52	81.50±0.70	70.25±0.81
1000	65.38±0.37	56.84±0.51	85.56±0.29	76.32±0.64
IC_{50}	**461.50**	**805.41**	**157.45**	**287.50**

Data represented as Mean values of three samples ± S.E.M.

Table No. 2A: Liver biomarkers of alcohol induced rats liver injury treated with methanolic extract of *Moringa oleifera* pods and Silymarin.

Group / Treatment	ALT (IU/L)		
	0th Day	26th Day	51th Day
Group A treated with 1% Sod.CMC	67.23± 1.44	57.15±1.95	62.19±3.04
Group B treated with Ethanol (3.76 g/kg)	58.52±2.04	211.32±4.71	219.25±2.01###
Group C treated with alcohol (3.7 g/kg) + MEMOP (100 mg/kg)	69.57±2.61	230.06±3.12	182.54±3.23***
Group D treated with alcohol (3.7 g/kg) + MEMOP (200 mg/kg)	64.76±2.98	229.73±7.10	125.37±4.07***
Group E treated with alcohol (3.7 g/kg) + MEMOP (400 mg/kg)	52.04±1.68	217.66±5.52	96.04±4.09***
Group F treated with alcohol (3.7 g/kg) + Silymarin (100 mg/kg)	59.73±2.92	224.06±2.61	78.14±3.43***

Table No. 2B: Liver biomarkers of alcohol induced rats liver injury treated with methanolic extract of *Moringa oleifera* pods and Silymarin.

Group / Treatment	AST (IU/L)			ALP (IU/L)		
	0th Day	26th Day	51th Day	0th Day	26th Day	51th Day
Group A treated with 1% Sod.CMC	82.23±0.70	84.06±2.31	85.81±2.62	159.17±2.46	145.71±3.02	147.04±3.67
Group B treated with Ethanol (3.76 g/kg)	84.20±0.75	325.81±4.05	301.17±5.36###	140.28±4.03	416.92±5.05	389.24±6.42###
Group C treated with alcohol (3.7 g/kg) + MEMOP (100 mg/kg)	84.22±0.68	339.26±4.05	265.22±5.95***	153.82±5.98	395.15±4.08	326.28±4.08***
Group D treated with alcohol (3.7 g/kg) + MEMOP (200 mg/kg)	83.95±1.93	340.11±6.89	202.29±6.48***	160.18±3.04	398.62±6.21	239.59±7.43***
Group E treated with alcohol (3.7 g/kg) + MEMOP (400 mg/kg)	87.20±0.59	329.21±4.09	112.62±5.98***	149.13±4.09	420.67±6.83	181.92±6.58***
Group F treated with alcohol (3.7 g/kg) + Silymarin (100 mg/kg)	90.93±1.09	336.34±4.06	98.59±3.38***	152.01±3.04	405.22±3.51	161.67±4.27***

Data represented as Mean values of six rats ± S.E.M; Where, # indicates P <0.05, ##indicates P<0.01 and ###indicates P<0.001 as compared Group B with normal control group (Group A);
*indicates P<0.05, **indicates P<0.01 and ***indicates P<0.001 as compared rest of groups with Group B treated with alcohol. **Note**: MEMOP: Methanolic extract of *Moringa Oleifera* pods.

Table No. 3: Liver biomarkers of alcohol induced rats liver injury treated with methanolic extract of *Moringa oleifera* pods and Silymarin.

Group/ Treatment	Total Bilirubin (mg/dl)			Total Protein (g/dl)		
	0th Day	26th Day	51th Day	0th Day	26th Day	51th Day
Group A treated with 1% Sod.CMC	0.28 ± 0.10	0.39 ± 0.04	0.32 ± 0.08	8.54 ± 0.29	8.61 ± 0.29	8.93 ± 0.49
Group B treated with Ethanol (3.76 g/kg)	0.34 ± 0.16	6.28 ± 0.46	5.79 ± 0.38###	8.94 ± 0.59	3.98 ± 0.11	3.31 ± 0.38###
Group C treated with alcohol (3.7 g/kg) + MEMOP (100 mg/kg)	0.27 ± 0.15	5.94 ± 0.39	4.08 ± 0.38**	8.09 ± 0.76	3.49 ± 0.48	4.92 ± 0.24*
Group D treated with alcohol (3.7 g/kg) + MEMOP (200 mg/kg)	0.28 ± 0.09	6.06 ± 0.32	2.78 ± 0.23***	7.86 ± 0.52	2.96 ± 0.52	5.83 ± 0.41**
Group E treated with alcohol (3.7 g/kg) + MEMOP (400 mg/kg)	0.31 ± 0.05	6.90 ± 0.52	1.03 ± 0.37***	8.04 ± 0.47	3.09 ± 0.47	7.21 ± 0.38***
Group F treated with alcohol (3.7 g/kg) + Silymarin (100 mg/kg)	0.30±0.14	6.54 ± 0.46	0.49 ± 0.20***	7.91 ± 0.64	3.55 ± 0.15	7.80 ± 0.26***

Data represented as Mean values of six rats ± S.E.M; Where, # indicates P <0.05, ##indicates P<0.01 and ###indicates P<0.001 as compared Group B with normal control group (Group A);
*indicates P<0.05, **indicates P<0.01 and ***indicates P<0.001 as compared rest of groups with Group B treated with alcohol. **Note**: MEMOP: Methanolic extract of *Moringa Oleifera* pods.

Table.No.4: Physical parameters of alcohol induced rats liver injury treated with methanolic extract of *Moringa oleifera* pods and Silymarin.

Group	Treatment	Curative study	
		Liver weight (g/100g)	Liver volume (ml/100g)
Group A	Treated with 1% Sod.CMC	3.46±0.08	3.94±0.05
Group B	Treated with Ethanol (3.76 g/kg)	7.08±0.12###	7.48±0.15###
Group C	Treated with alcohol (3.7 g/kg) + MEMOP (100 mg/kg)	5.73±0.09***	6.04±0.17***
Group D	Treated with alcohol (3.7 g/kg) + MEMOP (200 mg/kg)	4.38±0.18***	5.28±0.11***
Group E	Treated with alcohol (3.7 g/kg) + MEMOP (400 mg/kg)	4.04±0.16***	4.29±0.16***
Group F	Treated with alcohol (3.7 g/kg) + Silymarin (100 mg/kg)	3.71±0.14***	4.15±0.18***

Data represented as Mean values of six rats ± S.E.M; Where, # indicates P <0.05, ##indicates P<0.01 and ###indicates P<0.001 as compared Group B with normal control group (Group A); *indicates P<0.05, **indicates P<0.01 and ***indicates P<0.001 as compared rest of groups with Group B treated with alcohol. **Note**: MEMOP: Methanolic extract of *Moringa Oleifera* pods.

Table 5: Percentage protection produced by selected doses of *Moringa oleifera* pods and Silymarin against alcohol induced hepatotoxicity on 51th day.

S. No	Group/ Treatment	Percentage Protection						
		ALT	AST	ALP	Total.bil	Total. PR	Liver. wt	Liver. vol
1.	Group C Treated with alcohol (3.7 g/kg) + MEMOP (100mg/kg)	23.37	16.69	26.00	31.26	28.65	37.29	40.68
2.	Group D Treated with alcohol (3.7 g/kg) + MEMOP (200mg/kg)	59.77	45.91	61.79	55.03	44.84	74.59	62.15
3.	Group E Treated with alcohol (3.7 g/kg) + MEMOP (400mg/kg)	78.45	87.55	85.60	87.02	69.40	83.98	90.11
4.	Group F Treated with alcohol (3.7 g/kg) + Silymarin (100 mg/kg)	89.84	94.07	93.96	96.89	79.89	93.09	94.07

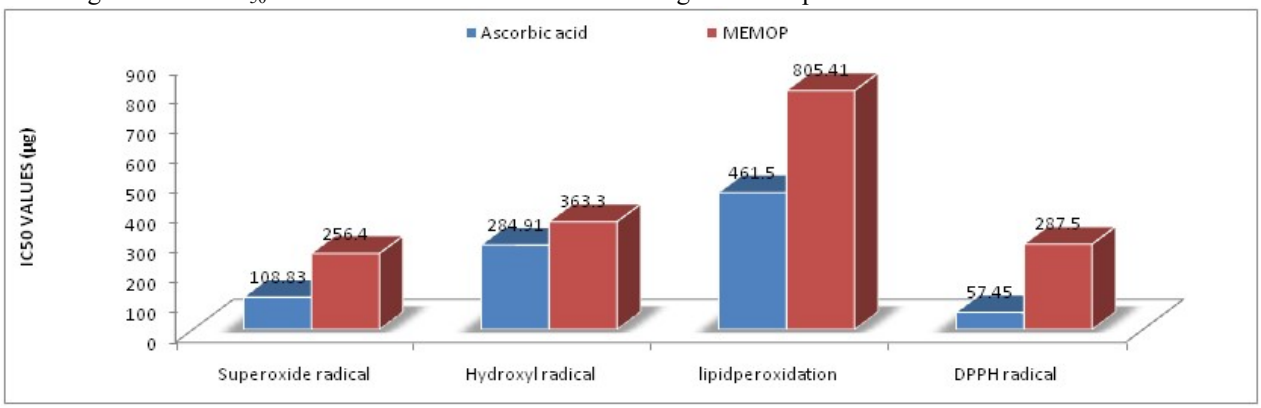
Note: MEMOP: Methanolic extract of *Moringa Oleifera* pods.

Table.No.6: Antioxidant parameters of alcohol induced rats liver injury treated with different doses of methanolic extract of *Moringa oleifera* pods and Silymarin.

Group	Treatment	Level of antioxidant parameters (Mean± SEM)			
		LPO Nmol / mg protein	SOD U/mg protein	Catalase U/mg protein	GPx µg/mg protein
Group A	Treated with 1% Sod.CMC	2.64±0.51	13.26±1.53	22.51±3.57	40.25±2.43
Group B	Treated with Ethanol (3.76 g/kg)	8.29±0.85###	4.28±0.84###	12.83±4.62n.s	21.08±5.79###
Group C	Treated with alcohol (3.7 g/kg) + MEMOP (100mg/kg)	7.24±0.59n.s	6.57±0.51*	15.49±5.76*	28.51±0.98n.s
Group D	Treated with alcohol (3.7 g/kg) + MEMO(200mg/kg)	5.61±0.47*	9.46±0.97*	18.91±1.09n.s	33.93±2.82*
Group E	Treated with alcohol (3.7 g/kg) + MEMOP(400mg/kg)	3.15±0.26***	12.05±1.07***	20.97±4.09*	38.94±1.61**
Group F	Treated with alcohol (3.7 g/kg) + Silymarin (100mg/kg)	2.97±0.38***	12.85±0.81***	21.07±2.41n.s	39.06±1.05**

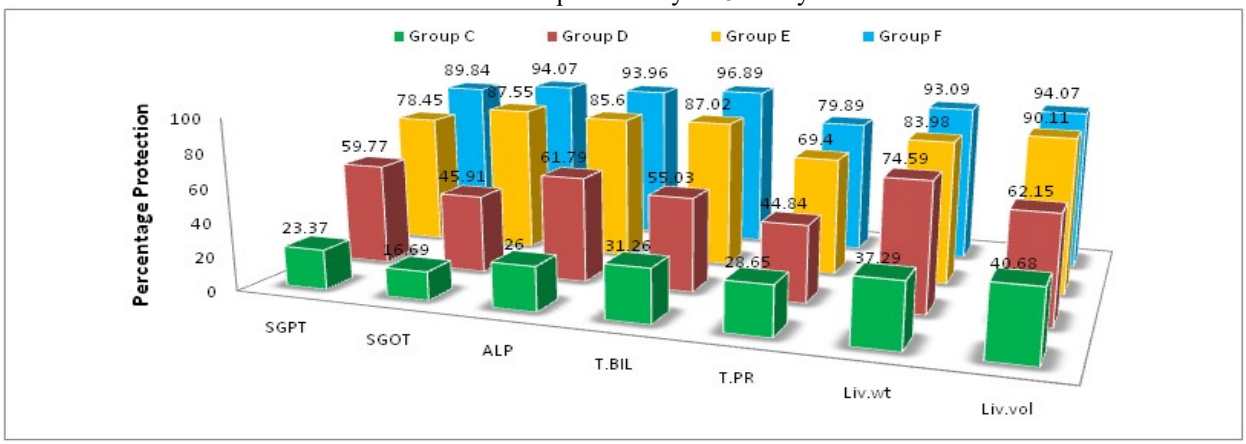
Data represented as Mean values of six rats ± S.E.M; Where, n.s indicates the not significant P>0.05; # indicates P <0.05, ##indicates P<0.01 and ###indicates P<0.001 as compared Group B with normal control group (Group A);*indicates P<0.05, **indicates P<0.01 and ***indicates P<0.001 as compared rest of groups with Group B treated with alcohol. Note: MEMOP: Methanolic extract of *Moringa Oleifera* pods.

Histogram .No.1: IC_{50} values of methanolic extract of Moringa Oleifera pods and ascorbic acid in four methods.



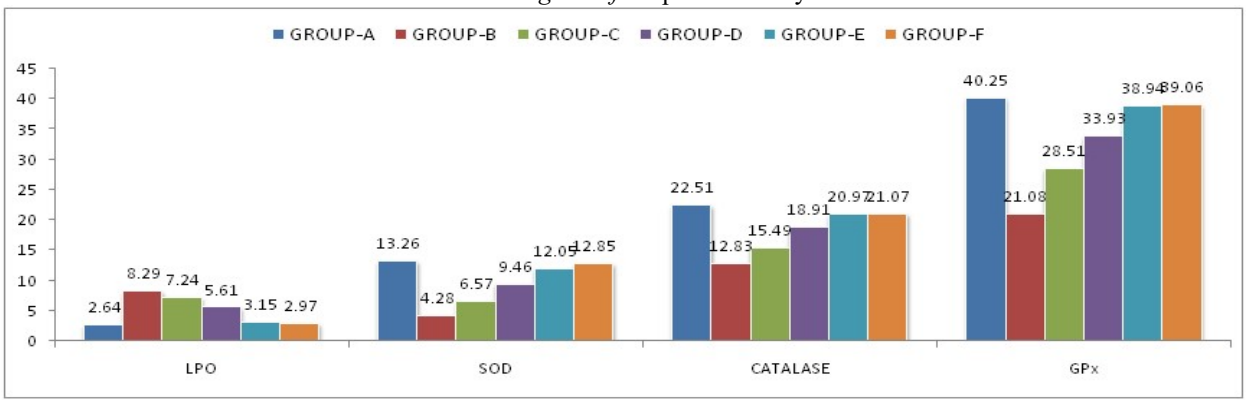
Note: MEMOP: Methanolic extract of *Moringa Oleifera* pods.

Histogram .No.2: Percentage protection produced by selected doses of *Moringa oleifera* pods and Silymarin against alcohol induced hepatotoxicity on 51th day.



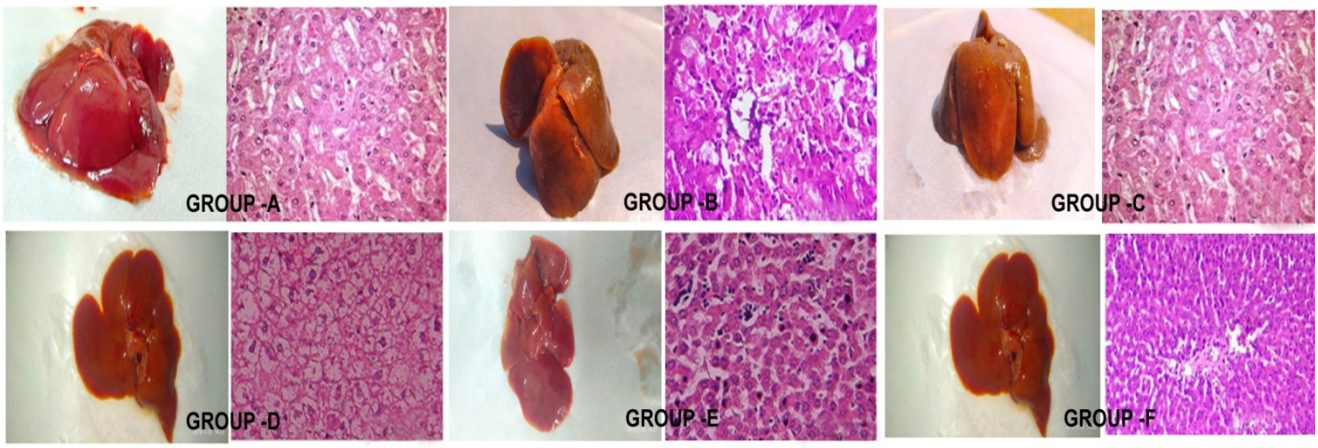
Note: **Group C**: Treated with 3.7 g/kg of alcohol and 100 mg/kg of MEMOP, **Group D**: Treated with 3.7 g/kg of alcohol and 200 mg/kg of MEMOP, **Group E**: Treated with 3.7 g/kg of alcohol and 400 mg/kg of MEMOP and **Group F**: Treated with 3.7 g/kg of alcohol and 100 mg/kg of Silymarin. MEMOP: Methanolic extract of *Moringa oleifera* pods.

Histogram.No.3: Antioxidant parameters of alcohol induced rats liver injury treated with different doses of methanolic extract of *Moringa oleifera* pods and Silymarin.



Note: **Group A**: Normal control, **Group B**: Treated with alcohol (3.7 g/kg), **Group C**: Treated with 3.7 g/kg of alcohol and 100 mg/kg of MEMOP, **Group D**: Treated with 3.7 g/kg of alcohol and 200 mg/kg of MEMOP, **Group E**: Treated with 3.7 g/kg of alcohol and 400 mg/kg of MEMOP and **Group F**: Treated with 3.7 g/kg of alcohol and 100 mg/kg of Silymarin. **MEMOP: Methanolic extract of *Moringa oleifera* pods.**

Figure.No.5: **A photomicrograph of hepatic tissue sections.**



Note: Group A: Negative control rat showed normal structure of hepatic tissue; **Group B)** An alcohol-control rat showed dilatation of many tubules with vacuolar degeneration, fatty ballooning, fatty infiltration, necrosis observed without regeneration**; Group F)** positive Silymarin control rat showed good recovery of hepatic tissue; **Group C**: Treated with 3.7 g/kg of alcohol and 100 mg/kg of MEMOP showed less protection; **Group D**: Treated with 3.7 g/kg of alcohol and 200 mg/kg of MEMOP showed reduction of vacuolar degeneration of hepatic cells but necrosis of cells still noticed; **Group E**: Treated with 3.7 g/kg of alcohol and 400 mg/kg of MEMOP showed recovery of hepatic tissue with regeneration cells. **MEMOP:** Methanolic extract of *Moringa oleifera* pods.

Acknowledgement

The authors are thankful to Hindu college of Pharmacy, Guntur for providing facilities.

Conflict of Interest: No conflict of interest found

Funding source: No funding source.

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